Comparative effects of inorganic phosphate and oxalate on uptake and release of Ca^{2+} by the sarcoplasmic reticulum in saponin skinned rat cardiac trabeculae

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1. Ventricular trabeculae from the right ventricle of rat heart were suspended in a 6 μl bath and 'skinned' with saponin (50 mg ml^{-1}). Preparations were perfused with solutions mimicking the intracellular milieu and the [Ca^{2+}] within the bath was monitored continuously using fura-2.

2. Application of 20 mM caffeine released Ca^{2+} from the sarcoplasmic reticulum (SR), resulting in a transient increase in the fura-2 fluorescence ratio. Caffeine-induced Ca^{2+} transients were smaller in the presence of 30 or 60 mM inorganic phosphate (P_i). This depressive effect of P_i on SR function was reversed by 10 mM creatine phosphate (CP). Caffeine-induced Ca^{2+} transients were also reduced in the presence of 10 mM oxalate, although this effect was not reversed by CP.

3. When perfusion was stopped in the presence of 30 or 60 mM P_i, the [Ca^{2+}] within the bath remained constant. However, when the flow was stopped in the presence of 60 mM P_i and 10 mM CP, a prolonged decrease in [Ca^{2+}] occurred, consistent with precipitation of calcium phosphate within the SR. A similar decrease in [Ca^{2+}] was observed when perfusion was stopped in the presence of 2 or 20 mM oxalate, in the absence or presence of CP.

4. The SR was Ca^{2+} depleted by withdrawal of ATP and exposure to 20 mM caffeine. Perfusion was then stopped and ATP reapplied, resulting in a maintained decrease in [Ca^{2+}] within the bath, due to SR Ca^{2+} uptake. Net Ca^{2+} uptake was markedly reduced in the presence of 30 mM P_i. In contrast, 20 mM oxalate increased Ca^{2+} uptake and the [Ca^{2+}] within the bath continued to fall over 2–3 min.

5. Introduction of P_i released Ca^{2+} from the SR. Ryanodine (100 μM) abolished caffeine-induced Ca^{2+} release while P_i-induced Ca^{2+} release was unaffected. P_i-induced Ca^{2+} release was reduced in the constant presence of 20 mM caffeine or 10 mM CP and was abolished completely by disruption of the SR membrane with Triton X-100. P_i-induced Ca^{2+} release occurred after abolition of SR Ca^{2+} uptake by ATP withdrawal.

6. These results suggest that the P_i-induced decrease in releasable Ca^{2+} does not result from precipitation of calcium phosphate within the SR lumen. P_i inhibits net SR Ca^{2+} uptake, but this appears to result from activation of a ryanodine-insensitive Ca^{2+} efflux pathway rather then inhibition of Ca^{2+} uptake. Possible mechanisms are considered, including reversal of the SR Ca^{2+} pump.

In cardiac muscle, a profound decrease in developed force occurs within the first few minutes of anoxia or global ischaemia. During this period, the intracellular concentration of P_i increases from about 2 to 20 mM due to breakdown of creatine phosphate (CP). Previous studies have shown that P_i decreases myofilament Ca^{2+} sensitivity and maximum Ca^{2+}-activated force (Kentish, 1986). More recent work suggests that P_i may also influence earlier steps in the excitation–contraction coupling process. In particular, experiments on skinned cardiac preparations have shown that millimolar levels of P_i markedly reduce the amount of Ca^{2+} available for release from the sarcoplasmic reticulum (SR; Zhu & Nosek, 1991; Smith & Steele, 1992; Steele, McAinsh & Smith, 1995). If this occurs when the cytosolic [P_i] rises during ischaemia or anoxia, the resulting decrease in systolic [Ca^{2+}] could contribute significantly to contractile failure.

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The mechanism underlying the inhibitory effect of P_i on the SR of skinned muscle fibres has not yet been established. It has recently been suggested that the decrease in releasable Ca^{2+} may result from calcium phosphate precipitation within the SR (Fryer, Owen, Lamb & Stephenson, 1995). Certainly, anions such as P_i and oxalate have been widely used in studies on isolated SR vesicles to enhance steady-state Ca^{2+} uptake. P_i enters the SR by diffusion where precipitation of calcium phosphate occurs if the solubility product is exceeded (Makinose & Hasselbach, 1965). Precipitation reduces the free [Ca^{2+}] within the SR, thereby increasing and prolonging Ca^{2+} uptake from the surrounding medium. In these circumstances, the decrease in free luminal [Ca^{2+}] would be expected to influence the amount of Ca^{2+} available for release from the SR. However, most previous studies on isolated SR vesicles have used levels of P_i in excess of 60 mM. The reduction in SR Ca^{2+} content reported in skinned cardiac preparations occurs at much lower [P_i], within the range known to occur during the early stages of anoxia or ischaemia (2–20 mM). It is not clear from previous work whether calcium phosphate precipitation occurs within the SR of skinned fibres at these lower levels of P_i, or whether precipitation contributes to the reduction of releasable Ca^{2+}.

The P_i-induced decrease in releasable Ca^{2+} could also be explained by inhibition of the SR Ca^{2+} pump or activation of a Ca^{2+} efflux pathway. Pump inhibition or increased SR Ca^{2+} permeability would reduce the amount of Ca^{2+} accumulated by the SR during a given loading period and there is evidence in support of both these mechanisms. In isolated SR vesicles, levels of P_i in the range expected to occur within the cytosol during anoxia have been shown to inhibit net Ca^{2+} uptake and the maximum SR ATPase rate (Zhu & Nosek, 1991). A number of early studies on isolated SR preparations showed that under some conditions, millimolar levels of P_i can also induce Ca^{2+} efflux by reversal of the Ca^{2+} pump (Hasselbach, 1978). Another possible efflux mechanism was identified in recent studies showing that P_i can directly activate SR Ca^{2+} channels isolated from both skeletal and cardiac muscle (Fruen, Mickelson, Shomer, Roghar & Louis, 1994; Kermode, Sitsapesin & Williams, 1995). In saponin skinned cardiac preparations, introduction of P_i is associated with a transient increase in [Ca^{2+}] which is consistent with P_i-induced SR Ca^{2+} efflux. However, the origins of the released Ca^{2+} and the efflux pathway have not yet been established in these preparations.

This study addresses the mechanism underlying the P_i-induced decrease in Ca^{2+} available for release from the SR in saponin-skinned cardiac preparations. A variety of protocols were used to assess (i) whether precipitation of calcium phosphate occurs within the SR under conditions relevant to anoxia or ischaemia and (ii) the influence of precipitation on releasable Ca^{2+}. The effects of P_i were compared with those of oxalate, which is also known to precipitate within the SR. In addition, the influence of P_i on net Ca^{2+} uptake by the SR and the mechanism of P_i-induced Ca^{2+} release were studied. The results suggest that the decrease in Ca^{2+} available for release from the SR in the presence of P_i does not involve precipitation of calcium phosphate within the SR. P_i inhibits net SR Ca^{2+} uptake, which may explain the reduction in caffeine-induced Ca^{2+} release. This action of P_i appears to result from activation of a ryanodine-insensitive Ca^{2+} efflux pathway.

METHODS

Sprague–Dawley rats (200–250 g) were killed by a blow to the head and cervical dislocation. Hearts were removed rapidly and bathed in Tyrode solution (Table 1). Free-running trabeculae 80–120 μm in diameter and 1–2 mm in length were dissected from the right ventricle. Preparations were permeabilized by exposure to solution A (Table 1) containing 50 μg ml^{-1} saponin for 30 min. Saponin treatment renders the sarcolemma permeable to small ions and molecules without disrupting SR function (Endo & Kitazawa, 1978). Saponin was then removed by washing the preparation in solution A before proceeding with the experiment. All experiments were done at room temperature (22–23 °C).

Apparatus for measurement of [Ca^{2+}] and tension

The apparatus for simultaneous measurement of tension and SR Ca^{2+} release is described in detail elsewhere (Smith & Steele, 1992). Briefly, trabeculae were attached between a tension transducer (Akers, Norway) and a fixed support, close to the surface of a coverslip. A Perspex column (5 mm diameter) was lowered to within 5–10 μm of the muscle to minimize the volume of the solution above the preparation. Throughout most experimental protocols, trabeculae were perfused by pumping solution at 1 ml min^{-1} via a narrow duct passing through the centre of the column. Waste solution was collected continuously at the column edge. The volume of solution between the coverslip and the base of the column (i.e. the effective bath volume) was approximately 6 μl. The perfusing solution could be changed using a series of valves, allowing the bath volume to be replaced within 10–15 s. Alternatively, solutions (with ATP or caffeine) could be rapidly applied via ducts close to the base of the column. A solenoid-controlled pneumatic system was used to deliver the same volume of solution (~100 μl) during each 50 ms application. Using this method, the caffeine or ATP concentration of the solution bathing the muscle was increased to 50% of the concentration injected within 8 ms.

The bath was placed on the stage of a Nikon Diaphot inverted microscope. The sarcomere pattern was viewed under ×40 magnification and sarcomere length adjusted to approximately 2·2 μm. Throughout the rest of the experiment, the muscle was viewed via a ×20 Fluor objective lens (Nikon CF Fluor; numerical aperture, 0·75). The preparation was alternately illuminated with light of wavelengths 340 and 380 nm at 50 Hz frequency using a spinning wheel spectrophotometer (Cairn Research, Faversham, Kent, UK). The average [Ca^{2+}] within the visual field containing the preparation was indicated by the ratio of light intensities emitted at >500 nm. Light emitted from areas of the field not occupied by the muscle image was reduced using a variable rectangular window on the side-port of the microscope.

Table 1. Solution composition (mM)

	A	B	C	D	E	F	G	H	Tyrode
K^+	130	130	130	130	130	130	130	130	5
Na^+	40	40	40	40	40	40	40	40	120
Mg^{2+}	6	6	8·4	8·4	11	11	1·5	1·9	1·5
Ca^{2+}	0·04	0·04	0·04	0·04	0·04	0·04	0·04	0·04	2
ATP	5	5	5	5	5	5	—	—	—
EGTA	0·2	0·2	0·2	0·2	0·2	0·2	0·2	0·2	—
Hepes	25	25	25	25	25	25	25	25	25
Cl^-	142	122	56·9	36·9	126	106	133	98·8	132
HPO_4^-	—	—	60	60	—	—	—	10	—
Oxalate	—	—	—	—	20	20	—	—	—
CP	—	10	—	10	—	10	—	—	—

In some experiments, 0·2 mM EGTA was replaced with 0·05 mM BAPTA, as indicated in the figures.

Solution composition and fluorescence measurements

Unless otherwise stated, all chemicals were obtained from Sigma. Basic solution compositions are given in Table 1. Most solutions were weakly Ca^{2+} buffered with 0·2 mM EGTA and contained 5 μM fura-2 (Calbiochem). In some experiments, the Ca^{2+} buffer capacity was further reduced by substitution of 0·05 mM BAPTA for EGTA. Azide (5 mM) was also included to inhibit possible mitochondrial activity. The equilibrium concentrations of metal ions were calculated using the affinity constants for H^+, Ca^{2+} and Mg^{2+} for EGTA taken from Miller & Smith (1984). The affinity constants for ATP and ADP are those quoted by Fabiato & Fabiato (1979). Corrections for ionic strength, details of pH measurement, allowance for EGTA purity and the principles of the calculations are given in Miller & Smith (1984). The free $[Mg^{2+}]$ was approximately 1·5 mM in all solutions, measured using Mag fura-2 (Calbiochem). Caffeine and ryanodine (Calbiochem) were added to each solution as required.

Under the conditions of this study, the apparent affinity constant of fura-2 for Ca^{2+} was 400 ± 20 nM ($n = 4$). This value was calculated by measuring the fluorescence ratio while perfusing the bath with strongly Ca^{2+}-buffered (10 mM EGTA) solutions over a range of $[Ca^{2+}]$. The free $[Ca^{2+}]$ of each solution shown in Table 1 was adjusted to 150 nM by addition of calcium chloride (1 M titration standard, BDH). While both tension and fura-2 fluorescence were simultaneously recorded in all experiments, only the fluorescence ratio is shown in most figures. The tension record was omitted due to the limitations of space (e.g. Fig. 1) or because the protocol produced no tension response. Cumulative data showing the effects of P_i on both Ca^{2+} and tension transients have been published elsewhere (Smith & Steele, 1992)

One limitation of the technique is that the skinned preparations are surrounded by solution containing fura-2, which contributes significantly to the signal. In addition, fura-2 binds to the trabeculae and continues to indicate $[Ca^{2+}]$. As considered previously, our estimates suggest that approximately 70% of the signal comes from the preparation (Smith & Steele, 1992). The fluorescence ratio can be readily calibrated (as described above) during constant perfusion of the bath when the $[Ca^{2+}]$ within the muscle is not changing. However, during a rapid transient release of Ca^{2+} from the SR, the peak fluorescence ratio is an underestimate of the $[Ca^{2+}]$ within the muscle. A similar situation occurs during rapid SR Ca^{2+} uptake. It is possible to correct the fluorescence ratio by measurement of the volume of solution surrounding each preparation, allowing more quantitative measurements to be made. However, this is not practical on a routine basis and in this study, bath volumes were not measured in each preparation. Due to the possibility of significant variation in the volume of solution surrounding each preparation, changes in the fluorescence ratio have been used to indicate qualitative rather than quantitative changes in $[Ca^{2+}]$.

Data recording and analysis

In most experiments, the ratio signal and individual wavelength intensities and the tension signals were lowpass filtered (−3 dB at 30 Hz) and recorded on tape for later analysis. In some experiments, where slow changes in $[Ca^{2+}]$ were being monitored, the filter frequency was decreased to 6 Hz. Data are presented as mean values ± S.D.

RESULTS

Precipitation in the presence of P_i and oxalate

Figure 1*A* shows fluorescence records from a saponin-treated trabecula. Throughout this protocol the preparation was suspended within a 6 μl bath, approximately 90–100 times the muscle volume. The upper panel shows records obtained during constant perfusion of the chamber with a weakly Ca^{2+}-buffered solution (solution A) at a rate of 1 ml min^{-1}. In this and all subsequent protocols, the free $[Ca^{2+}]$ of the perfusing solution was below that required to produce Ca^{2+}-activated force (~150 nM). The transient increases in the fluorescence ratio result from rapid application of 20 mM caffeine (arrowheads) and subsequent release of Ca^{2+} from the SR. The amplitude of the Ca^{2+} transients reflect the releasable pool of Ca^{2+} within the SR obtained under steady-state conditions, i.e. when further exposure to the bathing solution did not influence the amplitude of the caffeine-induced Ca^{2+} transient (see below). Representative steady-state Ca^{2+} transients are shown under control conditions (*a*), and following equilibration with solutions containing 30 mM P_i (*b*) or

60 mM P_i (*c*). The amplitude of the caffeine-induced Ca^{2+} transient was reduced in the presence of 30 and 60 mM P_i by 44 ± 3 ($n = 17$) and 56 ± 2·7% ($n = 4$), respectively (see Smith & Steele, 1992, for cumulated data at a range of $[P_i]$). We have shown previously that the depressive effect of 5–30 mM P_i on SR Ca^{2+} release is abolished by inclusion of CP in the perfusing solution (Steele *et al.* 1995). Figure 1*A d* shows that 10 mM CP also reverses the inhibitory effects of 60 mM P_i on SR Ca^{2+} release.

Figure 1*A* (lower panel) shows the protocol used to assess whether precipitation of calcium phosphate occurs within the SR at each $[P_i]$. The same trabecula was perfused for 10–15 min with each solution. Under control conditions, the releasable pool of Ca^{2+} within the SR reaches a steady state within 1–2 min (Steele, Orchard & Smith, 1992). Thereafter, net SR Ca^{2+} uptake ceases and when perfusion is stopped, the $[Ca^{2+}]$ within the bath remains constant (Fig. 1*A a*, lower panel). In the presence of P_i, precipitation

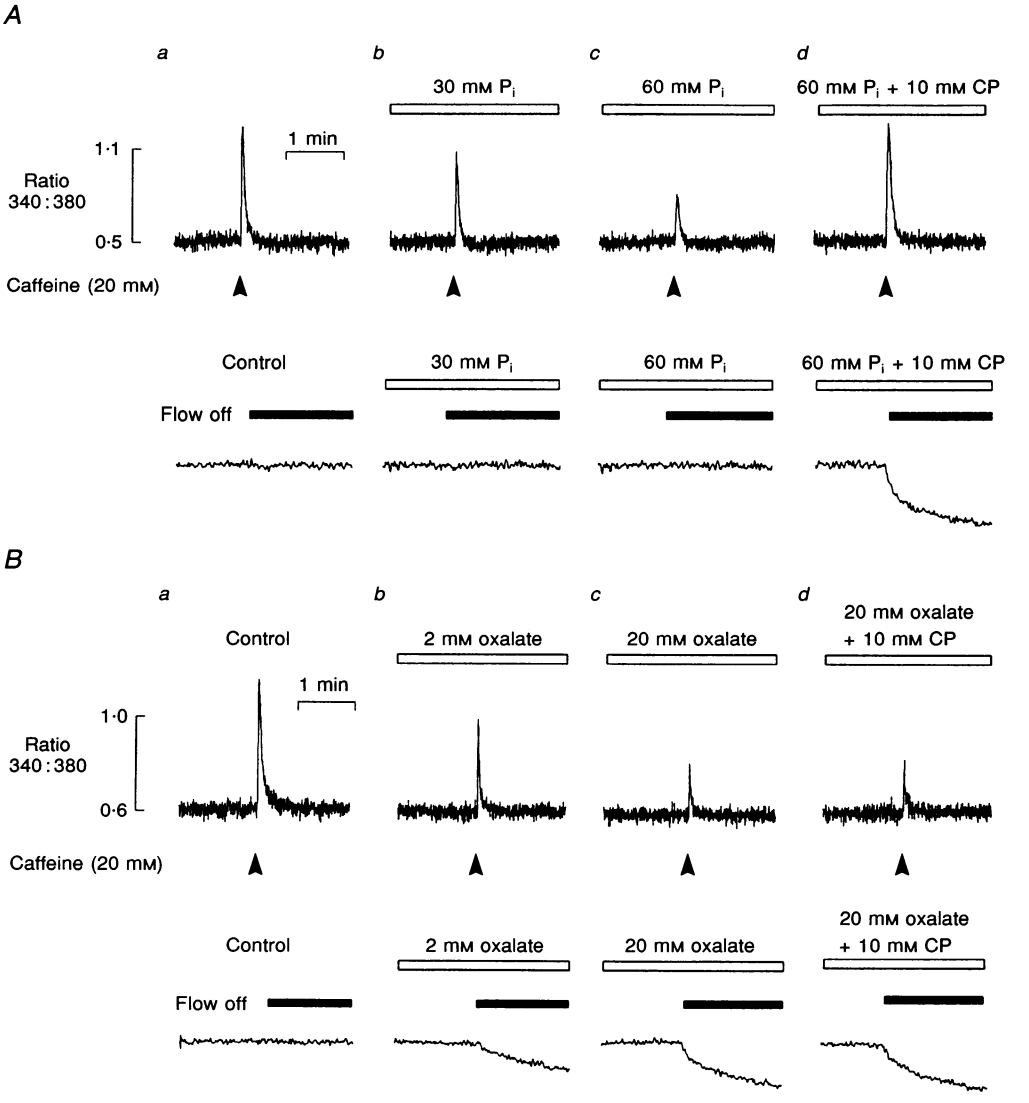

Figure 1. Effects of P_i or oxalate on caffeine-induced Ca^{2+} release

A, upper panel, caffeine-induced Ca^{2+} transients obtained during continuous perfusion of a saponin-treated trabecula. Each brief caffeine application (arrowhead) resulted in a transient increase in fluorescence ratio due to SR Ca^{2+} release. Steady-state responses are shown under control conditions (*a*), and in the presence of 30 mM P_i (*b*), 60 mM P_i (*c*) and 60 mM P_i with 10 mM CP (*d*). Lower panel, effects of stopping perfusion on bathing $[Ca^{2+}]$ under each condition (*a–d*). The lowpass filter cut-off frequency was decreased from 30 to 6 Hz. *B*, protocol repeated (in another preparation) in the presence of 2 and 20 mM oxalate. In *A* and *B*, the free $[Ca^{2+}]$ of the perfusing solutions was 150 nM. Control responses were obtained with solution A (Table 1). Concentrations of P_i lower than 60 mM were obtained by mixing solutions A and C. Oxalate concentrations lower than 20 mM were obtained by mixing solutions E and A.

will occur within the SR if the solubility product for calcium phosphate is exceeded. The continual entry of P_i into the SR followed by precipitation lowers the steady-state luminal $[Ca^{2+}]$, causing a maintained Ca^{2+} uptake (Makinose & Hasselbach, 1965). As the preparation occupies a significant volume of the bath, precipitation should result in a sustained decrease in the $[Ca^{2+}]$ of the surrounding solution when perfusion is stopped. However, when perfusion was stopped in the presence of 30 or 60 mM P_i, the $[Ca^{2+}]$ within the bath remained constant (Fig. 1*A b* and *A c*, lower panel). This suggests that precipitation does not occur within the SR under conditions where P_i reduces caffeine-induced Ca^{2+} release. In contrast, a sustained decrease in $[Ca^{2+}]$ consistent with precipitation did occur in the presence of 60 mM P_i and 10 mM CP (Fig. 1*A d*, lower panel). Similar results were obtained in four other preparations.

Figure 1*B* shows the same protocol carried out in the presence of oxalate; 2 and 20 mM oxalate reduced the amplitude of the caffeine-induced Ca^{2+} transients by $25{\cdot}7 \pm 6$ ($n = 4$) and $74{\cdot}6 \pm 9{\cdot}62\%$ ($n = 6$), respectively, during constant perfusion (Fig. 1*B b* and *B c*, upper panel). However, unlike the results obtained in the presence of P_i, inclusion of 10 mM CP in the perfusing solution had no significant effect on the caffeine-induced Ca^{2+} transients (Fig. 1*B d*, upper panel). Furthermore, when bath perfusion was stopped, precipitation of calcium oxalate was apparent at concentrations as low as 2 mM and in the absence of CP (Fig. 1*B b*, lower panel). At lower concentrations, where precipitation did not occur, caffeine-induced Ca^{2+} transients were not reduced in amplitude (not shown). In most preparations, CP had little or no effect on the rate that bathing $[Ca^{2+}]$ decreased during precipitation (Fig. 1*B d*, lower panel). Similar results were obtained in four other preparations. These results suggest that (unlike P_i) the decrease in caffeine-induced Ca^{2+} release in the presence of oxalate results from precipitation of calcium oxalate within the SR.

The sustained decrease in $[Ca^{2+}]$ observed when the bath perfusion was stopped did not occur if oxalate or P_i (and CP) was introduced after withdrawal of ATP. Furthermore, the rate of decrease was not influenced by mitochondrial inhibitors such as azide or cyanide. These observations suggest that the decrease in $[Ca^{2+}]$ results from ATP-dependent Ca^{2+} uptake by the SR and does not involve mitochondrial Ca^{2+} accumulation. The sustained decrease in $[Ca^{2+}]$ did not occur if the preparation was absent from the bath (not shown), confirming that the calcium phosphate or calcium oxalate solubility product is not exceeded in the basic perfusing solution. In general, the rate of decline of $[Ca^{2+}]$ slowed progressively after 1–2 min. This is expected, as the rate of Ca^{2+} accumulation will decrease as the $[Ca^{2+}]$ outside the SR falls further below the K_m of the Ca^{2+} pump. An additional factor which might influence the results when the flow is stopped for prolonged periods is that ATP depletion and accumulation of hydrolysis products (ADP and P_i) may inhibit net Ca^{2+} uptake. However, this factor does not appear to influence the SR as CP (which buffers ATP) had little effect on oxalate-supported Ca^{2+} uptake, minutes after the flow was stopped (Fig. 1*A d*). In any case, ATP breakdown and accumulation of hydrolysis products is unlikely to be significant in the first few seconds after the flow is stopped and any such effect would not qualitatively alter interpretation of the results.

Net SR Ca^{2+} uptake in the presence of P_i or oxalate

As considered earlier, another possible explanation for the decrease in Ca^{2+} available for release from the SR is that P_i might inhibit net Ca^{2+} uptake. The protocol shown in Fig. 2*A* was used to assess directly the effects of P_i and oxalate on net Ca^{2+} uptake by the SR. The preparation was initially perfused with a weakly Ca^{2+}-buffered solution (solution A). ATP was then withdrawn to abolish Ca^{2+} uptake by the SR ATPase (solution F) and caffeine (20 mM) simultaneously applied to release all of the available Ca^{2+}. This resulted in a transient release of Ca^{2+} from the SR (upper panel) and development of a sustained rigor contracture (lower panel). The muscle was then perfused with a caffeine-free solution for approximately 100 s. Thereafter, brief (50 ms) reapplication of caffeine failed to release Ca^{2+} from the SR, confirming that (i) the releasable pool of Ca^{2+} within the SR was depleted and (ii) SR Ca^{2+} uptake was abolished. The flow was then stopped and the solution changed (within 50 ms) to one with 5 mM ATP (solution A). This caused a rapid decrease in $[Ca^{2+}]$ due to Ca^{2+} uptake by the SR and a new steady-state level was approached after 2–3 min. The undershoot in the $[Ca^{2+}]$ probably occurred because the $[Ca^{2+}]$ measurements were restricted to the volume of the bath containing the preparation using a rectangular diaphragm (see Methods). When ATP was added, the $[Ca^{2+}]$ rapidly decreased within this volume due to SR Ca^{2+} uptake. However, as the SR approached its maximum capacity for Ca^{2+}, net uptake decreased to zero and the $[Ca^{2+}]$ equalized throughout the total bath volume, resulting in a slow rise to a new steady-state level. The difference between the final steady-state $[Ca^{2+}]$ and the $[Ca^{2+}]$ of the perfusing solution reflected the amount of Ca^{2+} accumulated by the SR under each condition.

The protocol shown in Fig. 2*A* was repeated in the presence of P_i and oxalate to assess the effects on net SR Ca^{2+} uptake (Fig. 2*B*). In each case, P_i or oxalate was introduced immediately after exposure to the 0 mM ATP–20 mM caffeine solution. In the presence of 30 mM P_i, net Ca^{2+} uptake by the SR was markedly reduced. In contrast, 20 mM oxalate increased the initial rapid phase of Ca^{2+} uptake. Thereafter, the $[Ca^{2+}]$ within the bath continued to decrease over 3–4 min. Again, the maintained phase of Ca^{2+} uptake is consistent with precipitation of calcium oxalate within the SR.

As in Fig. 1, a potential problem with this protocol is that initiation of SR Ca^{2+} uptake within the limited bath volume will result in a progressive decrease in [ATP] and

an increase in levels of hydrolysis products. This factor may influence the final [Ca^{2+}] within the bath achieved after several minutes. However, differences between the control record and those obtained in the presence of P_i or oxalate were apparent within 1–2 s after initiation of Ca^{2+} uptake. At this point, the mean [ATP] is unlikely to have changed significantly. These results provide direct evidence that P_i decreases net SR Ca^{2+} uptake and this effect may explain the fall in releasable Ca^{2+}(Fig. 1*A*, upper panel). In contrast, oxalate increases and prolongs net SR Ca^{2+} uptake, consistent with precipitation within the SR lumen.

Characteristics of P_i-induced Ca^{2+} release

While Fig. 2 shows that P_i reduces net Ca^{2+} accumulation by the SR, this could result from inhibition of Ca^{2+} uptake or activation of a Ca^{2+} efflux pathway. In previous studies, we have provided evidence that introduction of P_i is associated with a transient increase in the [Ca^{2+}] within skinned cardiac preparations (Smith & Steele, 1992). While this phenomenon probably results from P_i-induced Ca^{2+} release from the SR, the source of Ca^{2+} and the mechanism of release have not yet been studied. Figure 3 shows some characteristics of P_i-induced Ca^{2+} release in a saponin-treated trabecula. As in Fig. 1, the preparation was initially perfused with solution A, until the releasable pool of SR Ca^{2+} had reached a steady state. A typical P_i-induced Ca^{2+} release is shown in Fig. 3*A a*. On introduction of P_i, the [Ca^{2+}] within the trabecula transiently increased, reaching a peak after approximately 10 s. Withdrawal of P_i was associated with a transient decrease in [Ca^{2+}] which occurred with a similar time course. The mean amplitude of the P_i-induced Ca^{2+} transient was 23·2 ± 2·8% ($n = 8$) of that induced by a supramaximal concentration of caffeine.

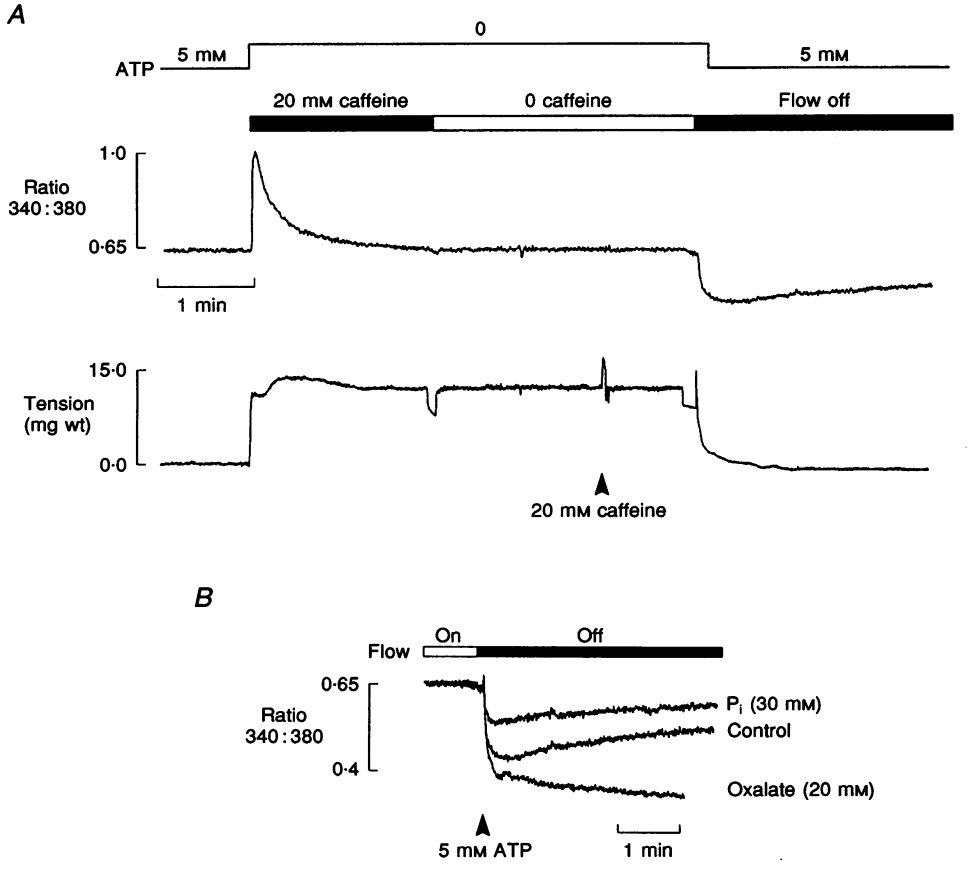

Figure 2. Effects of P_i and oxalate on net SR Ca^{2+} uptake

A, simultaneous records of fluorescence ratio (upper panel) and tension (lower panel) from a saponin-treated trabecula. Ca^{2+} was released from the SR (upper panel) and reuptake prevented by perfusion with a caffeine-containing solution, without ATP (solution G). Withdrawal of ATP also resulted in development of a maintained rigor contracture (lower panel). Caffeine (20 mM) was removed and 100 s later, brief (50 ms) reapplication (arrowhead) failed to release Ca^{2+}, confirming that the SR was Ca^{2+} depleted. The flow was then stopped and the bathing solution rapidly (within 50 ms) replaced with one containing 5 mM ATP (solution A), resulting in relaxation of the rigor response and a maintained decrease in [Ca^{2+}] due to SR Ca^{2+} uptake. *B*, superimposed records of ATP-induced SR Ca^{2+} uptake obtained under control conditions and in the presence of 30 mM P_i (obtained by mixing solutions A and C) or 20 mM oxalate (solution E). Throughout this protocol, the [Ca^{2+}] was weakly buffered with 0·05 mM BAPTA. All responses were obtained in the same saponin-treated trabecula.

If the source of Ca^{2+} is the SR, then interventions that deplete SR Ca^{2+} should reduce the P_i response. To investigate this possibility, the muscle was equilibrated with a solution containing caffeine (30 mM). Caffeine activates SR Ca^{2+} channels and should therefore reduce the amount of Ca^{2+} available for release. In the presence of caffeine (Fig. 3*A b*), P_i-induced Ca^{2+} release decreased markedly (by 67 ± 6%, $n = 6$). The transient fall in $[Ca^{2+}]$ on removal of P_i was similarly reduced. After removal of caffeine, the P_i-induced responses returned to control levels (Fig. 3*A c*). This effect of caffeine suggests that (i) most (if not all, see Discussion) of the Ca^{2+} released on addition of P_i is derived from the SR and (ii) P_i-induced Ca^{2+} release is proportional to the SR Ca^{2+} content.

The characteristic P_i-induced responses were also reduced by CP. Figure 3*B* shows P_i-induced responses obtained before (*a*) during (*b*) and after (*c*) exposure to 10 mM CP. The reduction in the P_i-induced Ca^{2+} release in the presence of CP occurs despite the fact that the Ca^{2+} content of the SR is the same as or greater than in control conditions (Fig. 1*A d*; see also Steele *et al.* 1995). This suggests that unlike caffeine, CP reduces P_i-induced Ca^{2+} release by inhibiting the release mechanism rather than altering the SR Ca^{2+} content. Similar results were obtained in four other preparations.

One possible mechanism which could explain P_i-induced Ca^{2+} release is direct activation of SR Ca^{2+} channels. This possibility was investigated by exposing the muscle to a high concentration of ryanodine (100 μM). At these levels, ryanodine blocks SR Ca^{2+} channels in the closed configuration, inducing complete insensitivity to all known activators (caffeine, adenine nucleotides, etc.). Figure 4*A* shows control Ca^{2+} transients obtained on addition of P_i (*a*) or caffeine (*b*). After treatment with 100 μM ryanodine, caffeine-induced Ca^{2+} release was completely abolished while P_i-induced release was unaffected (*c*). This suggests that P_i releases Ca^{2+} from the SR by a mechanism that does not involve activation of ryanodine-sensitive SR Ca^{2+} channels. Treatment of the same preparation with the non-ionic detergent Triton X-100 resulted in a prolonged release of Ca^{2+} due to disruption of the SR membrane (Fig. 4*B*). As expected, P_i failed to induce transient changes in $[Ca^{2+}]$ following membrane disruption. Similar results were obtained in six other preparations.

P_i-induced Ca^{2+} release in the absence of ATP

One possibility not yet addressed is that P_i-induced Ca^{2+} release might result from inhibition of the SR Ca^{2+} pump rather than activation of an efflux pathway. This could occur if steady-state Ca^{2+} uptake is balanced by a significant efflux. In these circumstances, rapid inhibition of Ca^{2+} uptake would result in *net* Ca^{2+} release. This possibility was investigated by introduction of P_i after withdrawal of ATP to abolish SR Ca^{2+} uptake (Fig. 5). The preparation was initially perfused with solution A until the releasable pool of SR Ca^{2+} had reached a steady state. The perfusing solution was then changed to one without ATP

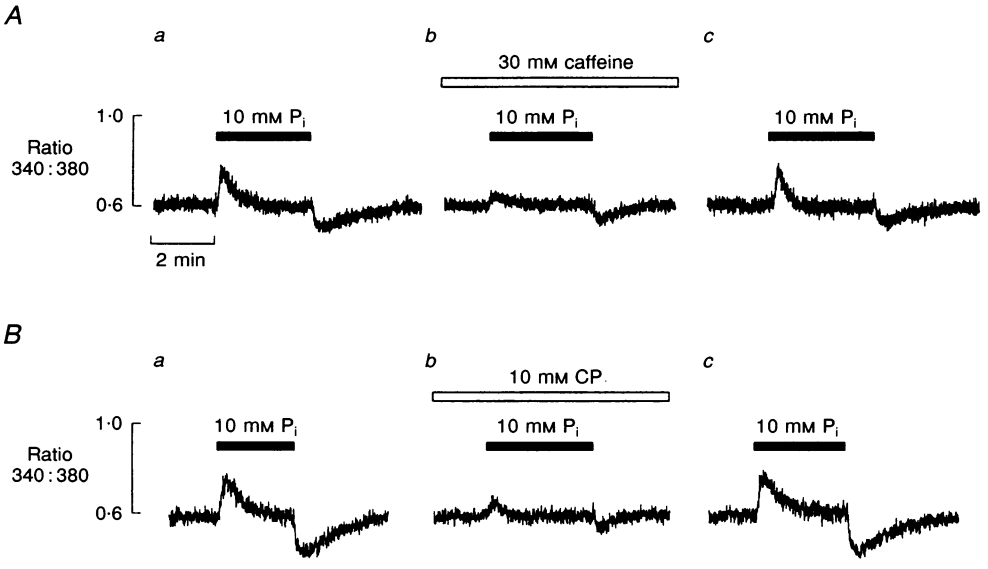

Figure 3. Effects of caffeine and CP on P_i-induced Ca^{2+} release

A, the trabecula was initially perfused with solution A. *A a* and *A c*, introduction of 10 mM P_i (obtained by mixing solutions A and C) induced a transient increase in $[Ca^{2+}]$ and removal of 10 mM P_i a transient decrease. *A b*, effect of equilibration with 30 mM caffeine on Ca^{2+} transients induced by introduction and withdrawal of 10 mM P_i. All responses were obtained in the same saponin-treated trabecula under conditions of steady-state Ca^{2+} loading. *Ba* and *Bc*, control P_i-induced Ca^{2+} transients. *Bb*, effect of equilibration with 10 mM CP (solution B) on Ca^{2+} transients induced by 10 mM P_i. All responses obtained in the same saponin-treated trabecula under conditions of steady-state Ca^{2+} loading.

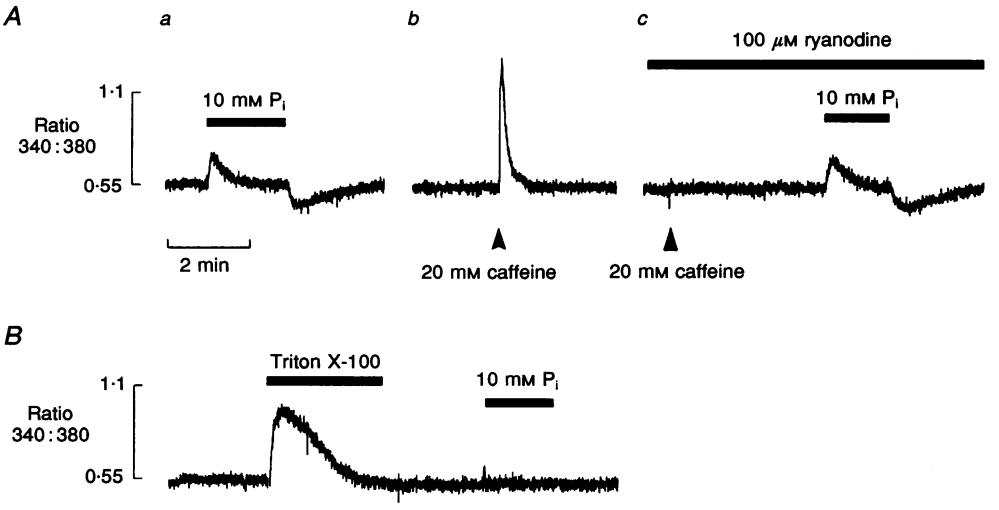

Figure 4. Effects of ryanodine on P_i-induced Ca^{2+} release

A a, transient increase in $[Ca^{2+}]$ induced by introduction of 10 mM P_i and decrease in $[Ca^{2+}]$ on P_i withdrawal. *A b*, SR Ca^{2+} release induced by brief application (arrowhead) of 20 mM caffeine. *A c*, ryanodine (100 μM) abolished caffeine-induced SR Ca^{2+} release while P_i-induced Ca^{2+} transients were unaffected. *B*, introduction of Triton X-100 induced a transient increase in $[Ca^{2+}]$ due to disruption of cellular membranes and abolished P_i-induced Ca^{2+} release. All responses were obtained in the same saponin-treated trabecula following equilibration with solution A.

(solution F). ATP withdrawal was associated with development of a maintained rigor contracture, indicating that the [ATP] had decreased below that required to support the activity of the myofilaments (not shown). Figure 5 shows a representative fluorescence record obtained approximately 5 min after ATP withdrawal. As in the presence of ATP, introduction of 10 mM P_i induced a transient release of Ca^{2+} from the SR. However, a transient decrease in $[Ca^{2+}]$ did not occur on removal of P_i, as expected if ATP is unavailable to support reuptake of Ca^{2+} by the SR (compare with Fig. 4*A a*). Brief (50 ms) application of 20 mM caffeine caused further release, confirming that P_i did not release all of the available Ca^{2+}. A second brief application of caffeine failed to release Ca^{2+} confirming that the SR was Ca^{2+} depleted. Thereafter, P_i-induced Ca^{2+} release was also absent. Qualitatively similar results were obtained following replacement of ATP with AMP-PCP (2 preparations) or adenosine (2 preparations).

Interpretation of results obtained using this protocol is complicated by several factors. Firstly, after cessation of Ca^{2+} uptake, the SR Ca^{2+} content decreases progressively

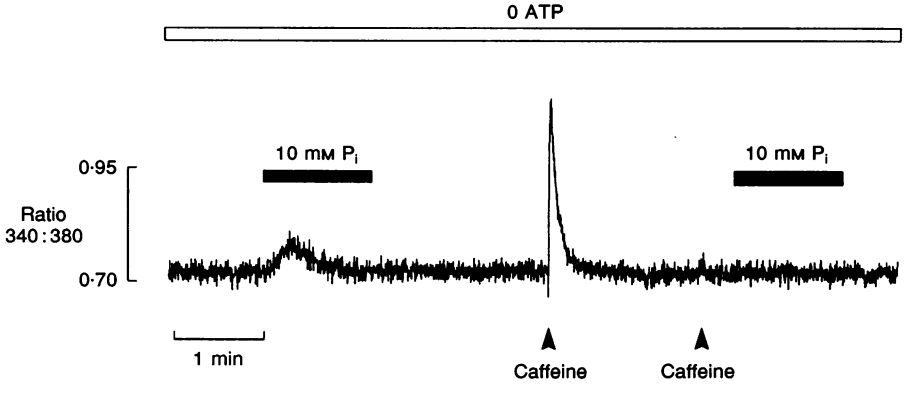

Figure 5. P_i-induced Ca^{2+} release in the absence of ATP

The preparation was equilibrated with solution A, prior to ATP withdrawal (solution G, not shown). P_i was then introduced (solution H) approximately 5 min after ATP withdrawal, resulting in a transient increase in $[Ca^{2+}]$. Subsequent brief (50 ms) application of 20 mM caffeine released Ca^{2+} from the SR. Reapplication of caffeine failed to release Ca^{2+}, confirming that the SR was Ca^{2+} depleted. Thereafter, P_i also failed to release Ca^{2+} from the SR.

due to passive Ca^{2+} efflux. Control experiments (not shown) suggest the rate of loss of Ca^{2+} from the SR occurs slowly over many minutes. Despite this, the $[Ca^{2+}]$ gradient across the SR membrane must be lower when P_i is introduced after withdrawal of ATP than under steady-state control conditions. This factor will tend to reduce P_i-induced Ca^{2+} release. Conversely, the inactivity of the Ca^{2+} pump in the absence of ATP will tend to increase the amplitude of the P_i-induced Ca^{2+} transient as competition between uptake and release will no longer occur. The relative influence of these effects is difficult to assess in any given preparation, making quantitative comparison of P_i-induced Ca^{2+} release in the absence and presence of Ca^{2+} uptake inappropriate. Despite these problems of interpretation, the fact that P_i-induced Ca^{2+} release occurs in the absence of ATP suggests that the release mechanism does not involve inhibition of the SR Ca^{2+} pump.

DISCUSSION

We have shown previously that P_i induces a concentration-dependent decrease in the amount of Ca^{2+} available for release from the SR in saponin-skinned cardiac muscle (Smith & Steele, 1992). The possible relevance of this phenomenon to contractile failure during myocardial ischaemia or anoxia has been discussed elsewhere (Steele *et al.* 1995) and will not be considered further. The present study addresses the mechanism underlying the inhibitory action of P_i on SR function, with particular reference to precipitation of calcium phosphate within the SR and possible effects of P_i on Ca^{2+} uptake and release. The protocols and results will be discussed with reference to Fig. 6, which illustrates the known Ca^{2+} flux pathways across the SR membrane including uptake by the Ca^{2+} pump (J_1) and efflux via Ca^{2+} channels (J_2), a passive leak pathway (J_3) and pump reversal (J_4; for review see Feher & Fabiato, 1990).

Experiments on isolated SR vesicles have shown that transport of Ca^{2+} across the SR membrane is initially coupled to ATP hydrolysis with a molar ratio of 2:1. However, as the luminal $[Ca^{2+}]$ rises, SR ATPase activity and Ca^{2+} uptake progressively decrease. Inhibition of pump activity by raised luminal $[Ca^{2+}]$ appears to result from occupation of low affinity Ca^{2+} binding sites at the inner surface of the SR membrane (e.g. Inesi & De Meis, 1989). The Ca^{2+} content of the SR rapidly approaches a steady state when uptake by the Ca^{2+} pump (J_1) is balanced by Ca^{2+} efflux. In Fig. 6 this corresponds to a situation where uptake (J_1) equals efflux ($J_2 + J_3 + J_4$) although, in the absence of P_i and Ca^{2+} channel activators, efflux should be small and occur predominantly via the passive leak pathway (i.e. $J_1 \approx J_2$). Once a steady state is achieved, and this takes approximately 1–2 min under the conditions of this study, there is no net Ca^{2+} accumulation from the surrounding fluid.

Steady-state Ca^{2+} fluxes are achieved under quite different conditions during precipitation of calcium phosphate (or calcium oxalate) within the SR. P_i enters the SR where precipitation will occur if the solubility product is exceeded. The resulting decrease in luminal $[Ca^{2+}]$ disinhibits the Ca^{2+} pump and this effect is maintained due to the continual entry of P_i from the surrounding medium. In these circumstances, steady-state uptake via the Ca^{2+} pump (J_1) exceeds efflux via the various pathways ($J_2 + J_3 + J_4$) and net Ca^{2+} uptake from the surrounding solution is greatly prolonged. In fact, this process is limited only by the eventual rupture of the SR membrane by calcium phosphate crystals (Feher & Lipford, 1995). While the steady-state free $[Ca^{2+}]$ is lower than under control conditions, the total SR Ca^{2+} content (free plus precipitate) increases progressively.

Precipitation of calcium phosphate and calcium oxalate within the SR

The protocol shown in Fig. 1 was used to assess whether precipitation of calcium phosphate within the SR underlies or contributes to the P_i-induced reduction in SR releasable Ca^{2+} reported in previous studies (Smith & Steele, 1992). All the results (Fig. 1*A* and *B*) were obtained after the releasable pool of Ca^{2+} within the SR had reached a steady state, i.e. further exposure to the bathing solution did not influence the caffeine-induced Ca^{2+} transient. Under these conditions, both P_i (Fig. 1*A*) and oxalate (Fig. 1*B*) induced a concentration-dependent reduction in the amount of Ca^{2+} released from the SR by caffeine. When the flow was stopped in the presence of oxalate, the $[Ca^{2+}]$ within the

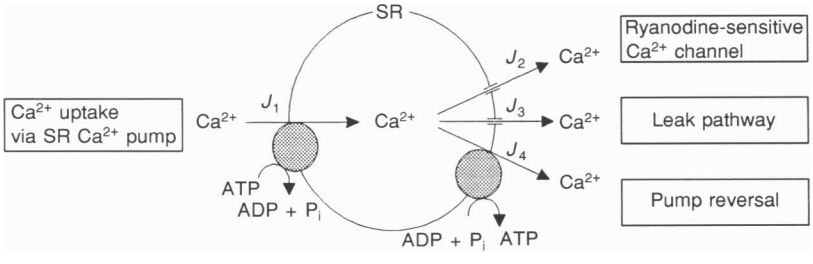

Figure 6. Diagram of SR Ca^{2+} flux pathways

Figure illustrates the known Ca^{2+} flux pathways across the SR including uptake via the Ca^{2+} pump (J_1) and efflux via Ca^{2+} channels (J_2), a passive leak pathway (J_3) and reversal of the Ca^{2+} pump (J_4).

bath decreased progressively over several minutes, consistent with precipitation of calcium oxalate within the SR. Precipitation can explain the decrease in releasable Ca^{2+} if it is assumed that (i) the free steady-state luminal $[Ca^{2+}]$ has been reduced and (ii) precipitated calcium oxalate is not available for release. However, there was no evidence of precipitation in the presence of 60 mM P_i, a concentration exceeding that known to occur within the cytosol during prolonged global ischaemia (38–40 mM; Allen & Orchard, 1987). The absence of precipitation suggests that some other action of P_i may reduce the Ca^{2+} content of the SR to such an extent that the solubility product for calcium phosphate is not exceeded. While P_i and oxalate both reduce the releasable pool of Ca^{2+} within the SR, the underlying mechanisms are clearly different.

Results consistent with precipitation of calcium phosphate were obtained in the presence of 60 mM P_i and 10 mM CP. Under these conditions, the amount of Ca^{2+} released by caffeine was the same as, or greater than, controls (Fig. 1*A d*). The ability of CP to reverse the P_i-induced decrease in caffeine-induced Ca^{2+} release has been addressed previously (Steele *et al.* 1995). Briefly, our interpretation of this effect is that CP reduces or abolishes an inhibitory action of P_i on SR function (see below). The steady-state luminal $[Ca^{2+}]$ can then increase to a high enough level for calcium phosphate precipitation to occur. It may seem paradoxical, however, that the amplitude of the caffeine-induced Ca^{2+} transient increases in the presence of 60 mM P_i and 10 mM CP (Fig. 1*A d*). As with oxalate, precipitation of calcium phosphate should lower the free $[Ca^{2+}]$ within the SR, which might have been expected to decrease caffeine-induced Ca^{2+} release. This apparent discrepancy probably reflects differences in the solubility products for calcium oxalate and calcium phosphate which are reported to be about 3 and 0·003 mM^2, respectively (Lide, 1995). Current estimates suggest that the free steady-state $[Ca^{2+}]$ within the SR is approximately 1–2 mM (Bers, 1991). Given these values, the luminal $[Ca^{2+}]$ might decrease sufficiently following application of caffeine to allow calcium phosphate precipitates to redissolve and then be released into the cytosol. It has been suggested that such a mechanism can also explain the P_i-induced increase in SR Ca^{2+} release in skinned skeletal muscle fibres (Fryer, Owen, Lamb & Stephenson, 1995). Release of precipitated calcium oxalate is less likely to occur because the luminal $[Ca^{2+}]$ would have to decrease by a much greater extent before precipitates could redissolve.

Effects of P_i and oxalate on net SR Ca^{2+} uptake

In the protocol shown in Fig. 2, the SR is first Ca^{2+} depleted and the Ca^{2+} pump stopped by withdrawal of ATP. On readdition of ATP, the pump is activated and Ca^{2+} is translocated from the cytosolic space into the SR. A steady state is rapidly approached (within 1–2 min) when uptake is exactly balanced by efflux. It is clear that net SR Ca^{2+} uptake is reduced markedly in the presence of P_i. While there are difficulties associated with quantification of these results, 30 mM P_i appears to decrease the amount of accumulated Ca^{2+} by approximately 50%. An effect of this magnitude could explain entirely the reduction in the caffeine-induced Ca^{2+} transient observed under similar conditions ($44 \pm 3\%$; $n = 17$). In contrast to the effects of P_i, oxalate increased and prolonged SR Ca^{2+} uptake, as expected if calcium oxalate precipitation is occurring within the SR.

Although P_i clearly inhibits net SR Ca^{2+} accumulation, this protocol provides little information regarding the underlying mechanism. This can be understood with reference to Fig. 6 where net Ca^{2+} uptake (under pre-steady-state conditions) is equal to the difference between uptake via the Ca^{2+} pump and efflux (i.e. $J_1 - (J_2 + J_3 + J_4)$). Inhibition of net Ca^{2+} uptake in the presence of P_i could result from pump inhibition, activation of a Ca^{2+} efflux pathway, or both. However, in this and previous studies, we have shown that introduction of P_i is associated with a net efflux of Ca^{2+} from the SR. From previously published data (e.g. Fig. 1*A* of Steele *et al.* 1995), it is possible to estimate from the area under the Ca^{2+} transients induced by caffeine and P_i that P_i-induced Ca^{2+} efflux can account for most if not all of the reduction in the releasable Ca^{2+}.

P_i-induced Ca^{2+} release

Figures 3 and 4 show several characteristics of P_i-induced Ca^{2+} release. When the preparation was equilibrated with a solution containing 30 mM caffeine, P_i-induced Ca^{2+} release was markedly reduced relative to preceding and succeeding control responses (Fig. 3*A*). P_i-induced Ca^{2+} release was decreased to a similar extent in the presence of CP (Fig. 3*B*). However, despite the apparent similarity in effect, it is unlikely that caffeine and CP act via a common mechanism. Caffeine activates SR Ca^{2+} channels, resulting in a decrease in luminal $[Ca^{2+}]$ which will continue until a new steady state is reached. Under these conditions, P_i-induced release would also be expected to decrease due to the reduced $[Ca^{2+}]$ gradient across the SR membrane. The fact that 30 mM caffeine does not completely abolish P_i-induced Ca^{2+} release is consistent with a recent study showing that the $[Ca^{2+}]$ gradient is not completely dissipated under these conditions (Smith, McAinsh & Steele, 1995). In contrast to the effects of caffeine, the Ca^{2+} content of the SR is unaltered by introduction of 10–15 mM CP alone (Steele *et al.* 1995). Furthermore, in the presence of CP and P_i, caffeine-induced Ca^{2+} release is unaffected or slightly increased (Fig. 1*A d*). Together, these results suggest that the decrease in P_i-induced Ca^{2+} release in the presence of CP reflects inhibition of the Ca^{2+} release process, rather than a change in the amount of Ca^{2+} available for release from the SR.

The effects of 100 μM ryanodine suggest that P_i and caffeine release SR Ca^{2+} by different mechanisms (Fig. 4). At this concentration, ryanodine blocks SR Ca^{2+} channels in the closed configuration, resulting in insensitivity to all

known activators. As flux through SR Ca^{2+} channels is expected to be small under control conditions, ryanodine treatment should have little effect on the $[Ca^{2+}]$ gradient across the SR membrane. Consistent with previous results on skinned fibres (Fabiato, 1985), caffeine-induced Ca^{2+} release was abolished by exposure to ryanodine. In contrast, P_i-induced Ca^{2+} release was not significantly affected. This suggests that P_i-induced Ca^{2+} release (i) does not result from direct activation of the SR Ca^{2+} channel and (ii) is not amplified by the Ca^{2+}-induced Ca^{2+} release mechanism associated with the SR Ca^{2+} channel.

Possible mechanisms underlying the effects of P_i in the absence and presence of CP

While the mechanism underlying P_i-induced Ca^{2+} efflux remains uncertain, the present results on skinned fibres appear consistent with studies on isolated SR vesicles. In these preparations, it was shown that millimolar levels of P_i can induce a rapid Ca^{2+}efflux from the SR by reversal of the Ca^{2+} pump (Barlogie, Hasselbach & Makinose, 1971), which is accompanied by synthesis of ATP from ADP (Makinose & Hasselbach, 1965, 1971; Deamer & Baskin, 1972; Suko, Hellmann & Winkler, 1977; Winkler & Suko, 1977). Pump reversal requires both micromolar levels of ADP and a $[Ca^{2+}]$ gradient across the SR membrane which provides the energy for ATP synthesis (Hasselbach, 1978).

In saponin-treated trabeculae, the mean [ADP] has been calculated to be approximately 60 μM, based on ATPase rates measured under similar conditions (Smith & Steele, 1992). This is close to the optimal [ADP] necessary for P_i-induced Ca^{2+} efflux via the Ca^{2+} pump (Barlogie *et al.* 1971). Therefore, the transient increase in $[Ca^{2+}]$ on addition of P_i (e.g. Fig. 4*A a*) may result from the dual action of P_i and ADP (produced by cellular ATPases) which together induce a net Ca^{2+} efflux via the SR Ca^{2+} pump. This would in turn reduce the Ca^{2+} content of the SR and the amplitude of the caffeine-induced Ca^{2+} transient. Activation of SR Ca^{2+} efflux may explain why calcium phosphate precipitation was not observed, even in the presence of 60 mM P_i (Fig. 1*A c*). Because the SR $[Ca^{2+}]$ content decreases progressively as the $[P_i]$ increases, the solubility product may never be exceeded under these circumstances.

The effects of CP are also consistent with P_i-induced pump reversal. It has been shown that inclusion of the ATP regenerating system phosphenolpyruvate and pyruvate kinase markedly reduces P_i-induced Ca^{2+} efflux in SR vesicle preparations (Soler, Teruel, Fernandez-Balda & Gomez-Fernandez, 1990). This occurs because rephosphorylation of ADP to ATP decreases the [ADP] sufficiently to limit ATP synthesis by the Ca^{2+} pump. In saponin-treated preparations, creatine phosphokinase is retained after saponin treatment (e.g. Steele, McAinsh & Smith, 1995). Therefore, addition of CP alone may reduce the [ADP] within the preparations sufficiently to inhibit P_i-induced Ca^{2+} efflux via pump reversal. When Ca^{2+} efflux is prevented or reduced by CP, the free luminal $[Ca^{2+}]$ increases towards normal levels. Under these conditions, precipitation of calcium phosphate can occur if the $[P_i]$ is sufficiently high (e.g. Fig. 1*A d*). The reduction in P_i-induced Ca^{2+} release in the presence of caffeine and the insensitivity to ryanodine are also consistent with reversal of the Ca^{2+} pump.

Relationship to previous studies on skinned preparations

In a recent study on mechanically skinned skeletal muscle fibres it was suggested that the inhibitory effects of P_i on cardiac and skeletal muscle SR can be explained entirely by precipitation of calcium phosphate within the SR (Fryer *et al.* 1995). However, the present results provide evidence that the reduction in releasable Ca^{2+} in the presence of P_i, does not result from precipitation of calcium phosphate within the SR, at least in cardiac muscle. While precipitation does occur in the presence of millimolar levels of CP and high levels of P_i, the physiological relevance of this is unclear. During the early stages of anoxia or ischaemia in cardiac muscle, the increase in $[P_i]$ is accompanied by a progressive fall in [CP]. We have previously addressed the effects of reciprocal changes in $[P_i]$ and [CP] on the SR of skinned cardiac preparations (Steele *et al.* 1995). Similar studies have yet to be done on skeletal muscle and the importance of P_i as a contributing factor to fatigue remains uncertain.

The interaction between CP and P_i at the level of the SR can also explain apparent discrepancies between the present study and previous work on skinned skeletal fibres (Steinen, Grass & Elzinga, 1993; Fryer *et al.* 1995). Zhu & Nosek (1991) studied the effects of complex changes in the intracellular milieu similar to those that occur during hypoxia. While it was found that P_i influenced SR function, the direction of the effect depended on the Ca^{2+} loading period. At short loading times, P_i decreased the SR Ca^{2+} content while high $[P_i]$ or long loading times increased the Ca^{2+} content. This can be explained by the presence of a low level of CP (1·4 mM) in the solutions which would be expected to only partially inhibit the effects of P_i on pump reversal (see Fig. 3 of Steele *et al.* 1995). The reduction in SR Ca^{2+} content observed at short loading times might therefore result from pump reversal. However, at longer loading periods, the luminal $[Ca^{2+}]$ may increase sufficiently to exceed the calcium phosphate solubility product, resulting in precipitation of calcium phosphate and an increase in the releasable Ca^{2+} (as suggested by the authors).

Conclusions

These results suggest that the P_i-induced decrease in Ca^{2+} available for release from the SR does not result from precipitation of calcium phosphate within the SR lumen. P_i inhibits net SR Ca^{2+} uptake but this appears to result from activation of a ryanodine-insensitive Ca^{2+} efflux pathway rather then inhibition of Ca^{2+} uptake *per se*. The results are consistent with previous studies demonstrating that P_i can induce Ca^{2+} efflux by reversal of the SR Ca^{2+} pump.

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